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Effect of Disinfectants on Pathogenic Free-Living Amoebae: in Axenic Conditions

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The amoebicidal properties of chlorine, chlorine dioxide, ozone, and deciquam 222 were examined in axenic conditions. *Naegleria* spp. were found to be more sensitive to chlorine and chlorine dioxide than *Acanthamoeba* spp. No marked difference in sensitivity to ozone or deciquam 222 could be detected between the pathogenic (A-1) and nonpathogenic (1501) strains of *Acanthamoeba* and the pathogenic (MsT) and nonpathogenic (P1200f) strains of *Naegleria*. Methods of disinfection are discussed with reference to suitability of the disinfectants to real conditions.

The finding of pathogenic free-living amoebae (PFLA) in chlorinated domestic and swimming waters (2, 4, 5, 10, 14) has led to an expression of concern by public health authorities over the possible contraction of primary amoebic meningoencephalitis via these waters. Cerva (4), after a review of 16 fatal cases of primary amoebic meningoencephalitis due to the use of an indoor chlorinated swimming pool, stated that, "the constant presence of numerous populations of the limax group *of amoebae* cannot be prevented even under the strictest observations of all routine safety measures applied to water systems of swimming pools." In 1972, Anderson and Jamieson (2) reported a case of primary meningoencephalitis in South Australia, the victim having playfully submerged his head in domestic bath water. They also reported that superchlorination to 10 mg·liter^{-1} had failed to eradicate *Naegleria* from a contaminated pool. Subsequently, Derreumaux et al. (11) demonstrated that 0.5 mg of HOCl, the active disinfecting component of chlorine disinfection, per liter was able to eradicate both *Naegleria* and *Acanthamoeba* spp. De Jonckheere and van de Voorde (10) found that an initial concentration of chlorine between 0.5 and 1.0 mg·liter^{-1} was cysticidal for *Naegleria* spp. but that *Acanthamoeba culbertsoni* cysts were not inactivated by 40 mg·liter^{-1}.

The present study was initiated to assess the effectiveness of chlorine, chlorine dioxide, ozone, and deciquam 222 against pathogenic and nonpathogenic species of *Naegleria* and *Acanthamoeba*.

MATERIALS AND METHODS

Culture of amoebae. *Naegleria gruberi* (P1200f) and *Naegleria fowleri* (MsT) were obtained from the National Health Institute, Wellington, New Zealand. *A. culbertsoni* (A-1) was supplied by the Culture Center for Algae and Protozoa, Cambridge, England, and *Acanthamoeba castellanii* (1501) was obtained from E. Willaert of the Prins Leopold Institute of Tropical Medicine, Antwerp, Belgium. Both *Naegleria* spp. were cultivated axenically in CYM medium, which consists of (wt/vol) 1.0% Casitone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 1.0% glucose, and 1.0% vitamin mix containing, per liter, 1.0 mg of thiamine hydrochloride, 0.2 mg of *d*-biotin, and 1.0 μg of vitamin B_{12}. The medium was supplemented with 20.0 cm^3 of bovine serum per liter, 90.0 mg of L-methionine per liter, 4.0 mg of hemin per liter, and 2 × 10^5 U of penicillin-streptomycin per cm^3 (9, 16). Both *Acanthamoeba* spp. were cultivated axenically in 4.0% Neff medium, which consists of (wt/vol) 4.0% protease-peptone (Difco), 0.75% yeast extract (Difco), 1.5% glucose, 1.0 mg of vitamin B_1 hydrochloride per liter, and 1.0 μg of vitamin B_{12} per liter (19).

Production of disinfectants. (i) Chlorine. A stock solution of sodium hypochlorite (British Drug Houses Ltd., Poole, England) was diluted to the appropriate concentration with sterile, chlorine-free, deionized water.

(ii) Chlorine dioxide. Chlorine dioxide was produced as recommended by the American Public Health Association (1).

A total of 5.0 g of sodium chlorite was dissolved in 375.0 cm^3 of chlorine-free, deionized water and placed in a flask. A total of 1.0 cm^3 of concentrated H_2SO_4 was added to 9.0 cm^3 of deionized water, mixed, and placed in a funnel above the flask. A smooth current of air was passed through the flask, and 5.0-cm^3 increments of the H_2SO_4 were introduced into it at 5.0-min intervals. The airflow was continued for a further 30.0 min, and the resulting solution was stored in a brown bottle at 4°C.

(iii) Ozone. Ozone was produced by the electrical discharge of oxygen. This involved passing dried oxygen through an Mk II ozonizer (British Oxygen Cryoproducts, London, England) and collecting the dissolved ozone in sterile 0.01 M phosphate buffer at pH 7.0.

(iv) Deciquam 222. Deciquam 222 (de-decyldimethyl-ammonium bromide) was donated by Maui Brothers, Auckland, New Zealand.

Chemical analysis of disinfectants. Chlorine, chlorine dioxide, and ozone were analyzed by the diethyl-*p*-phenylene diamine method, which uses diethyl-*p*-phenylene diamine as an indicator solution and is much preferred over other acid or neutral orthotolidine methods for its sharper chlorine-chloramine differentiation (18). Due to the lack of a reliable analytical test, no analyses of deciquam 222 were done.

Disinfectant testing. Amoebae were centrifuged from the culture media at 425 × *g* for 10 min and suspended in sterile 0.01 M phosphate buffer (pH 7.0). They were then washed three times in the same buffer, counted with a modified Fuchs-Rosenthal hemocytometer, and resuspended to a final concentration of 1.2×10^6 cells per cm^3. A viable count was then done with the plaque formation method (8, 12).

For the test, 24.6 cm^3 of the phosphate buffer containing the required concentration of any one of the disinfectants under examination was dispersed into 250-cm^3 flasks. A total of 0.4 cm^3 of the washed amoebae was added to each flask, and the flasks were incubated for 30 min at 25°C. Disinfectant levels were assayed at the commencement of incubation and after 30 min of incubation on 10 cm^3 of the test culture. At that point, a crystal of sodium thiosulfate was added to neutralize chlorine, chlorine dioxide, or ozone, whereas deciquam 222 was neutralized with neutral red calcium chloride. The remaining 25 cm^3 was filtered through a 5-μm cellulose-acetate filter. The filter was then washed with 5 cm^3 of sterile Page amoeba saline (17), and 0.1 cm^3 was diluted and plated onto a lawn of *Enterobacter cloacae* on NM agar (12).

The resulting plaques, which were formed by the amoebae feeding on the bacteria and leaving a clear area, were counted and related to the original sample to indicate the number of viable amoebae present.

RESULTS

Chlorine. The amoebicidal capacity of chlorine is shown in Table 1. Because the deionized water exerted a negligible chlorine demand, the total available chlorine at zero time was equal to the free available chlorine (FAC) at the same time. It can be seen that *Naegleria* spp. were more sensitive to chlorine than *Acanthamoeba* spp., with 0.79 mg of total available chlorine or initial FAC per liter being amoebicidal for *Naegleria* spp., as opposed to 1.25 mg of total available chlorine per liter for *Acanthamoeba* spp. The difference, (total available chlorine – FAC), known as combined available chlorine, represents the chlorine demand which was due to the organic content of the inoculum, i.e., amoebae, reacting with the chlorine during the 30-min contact time.

Disinfectants other than chlorine. Table 2 shows the comparative amoebicidal capacity of chlorine dioxide, ozone, and deciquam 222. Whereas *Naegleria* spp. were once again more sensitive to the chlorine-containing disinfectant as compared with *Acanthamoeba* spp., no

TABLE 1. *Amoebicidal capacity of chlorine*

Species (strain)	pH	Temp (°C)	Concn of amoebae × 10^4 per cm^3 at time 0	Chlorine level ($mg \cdot liter^{-1}$) for[a]			Survivors after 30 min	
				TAC = FAC at time 0	FAC after 30 min	CAC = chlorine demand	No. of amoebae per cm^3	%
N. gruberi (P1200f)	7.0	25	1.92	0.79	0.16	0.630	0	0
				0.675	0.125	0.50	1	0.005
				0.625	0.125	0.50	10	0.052
				0.625	0.125	0.50	15	0.078
				0.55	0.105	0.45	26	0.14
N. fowleri (MsT)	7.0	25	1.92	0.925	0.275	0.650	0	0
				0.750	0.175	0.575	0	0
				0.74	0.19	0.55	0	0
				0.625	0.125	0.50	7	0.036
				0.575	0.125	0.55	18	0.093
A. castellanii (1501)	7.0	25	1.92	1.10	0.25	0.85	0	0
				1.02	0.22	0.80	0	0
				1.0	0.25	0.75	2	0.01
				0.85	0.20	0.65	10	0.052
				0.80	0.175	0.625	17	0.089
A. culbertsoni (A-1)	7.0	25	1.92	1.25	0.25	1.0	0	0
				1.25	0.25	1.0	0	0
				1.09	0.14	0.95	23	0.12
				0.95	0.20	0.75	18	0.093

[a] TAC, Total available chlorine; CAC, combined available chlorine.

marked difference in the sensitivity among the four strains (MsT, P1200f, A-1, and 1501) toward ozone or deciquam 222 could be detected. Of the three alternative disinfectants examined, deciquam 222 exhibited the greatest amoebicidal capacity, followed by chlorine dioxide and ozone.

DISCUSSION

Disinfection can be defined as "the killing of the larger portion (but not necessarily all) of the harmful and objectionable microorganisms in, or on, a medium by means of chemicals, heat or ultraviolet light, etc." (22). Thus, it is not to be confused with sterilization.

The results demonstrate that all four disinfectants examined (chlorine, chlorine dioxide, deciquam 222, and ozone) possess amoebicidal properties and would be suitable for the disinfection of waters contaminated with PFLA. However, the ultimate choice of a particular disinfectant remains closely tied to the chemical and physical characteristics of the water to be treated and the particular properties of the disinfectant (Table 3).

Since chlorine was introduced into water treatment nearly 80 years ago, it has become almost the only method used for the active disinfection of potable water supplies (24). This predominant position has been gained because of its potency and range of effectiveness as a germicide; its ease of application, measurement, control, and economy; its relative freedom from toxic or physiological effects; and its reasonable persistence in waters. With a batch system, in which only a single dose of chlorine is added, Table 1 shows that initial concentrations of 0.74, 0.79, 1.0, and 1.25 mg·liter^{-1} had a sterilizing effect on *N. fowleri*, *N. gruberi*, *A. castellanii*, and *A. culbertsoni*, respectively. It is thought that the difference in susceptibility to chlorine between the two genera is more likely to be a consequence of the difference in chemical (especially protein) composition of the cell membranes than of the difference in metabolism.

The use of chlorine as a disinfectant for PFLA was reported by Anderson and Jamieson (2), who failed to eliminate *N. fowleri* which had been superchlorinated with 10.0 mg of chlorine per liter. However, the chlorine demand of the water was not tested, and thus there may have been insufficient FAC available for disinfection. With a batch method but pH and concentrations of amoebae different from those used in this study, Derreumaux et al. (11) demonstrated that water with an initial FAC content of 1.4 mg of chlorine per liter was able to sterilize 2×10^3 trophozoites per cm^3 in 30 min. This agrees well with the figures reported in Table 1, i.e., 1.25 mg of chlorine per liter sterilized 1.92×10^4 trophozoites per cm^3 in 30 min. They also reported *Naegleria* to be more sensitive to chlorine than *Acanthamoeba*, as did De Jonckheere and van de Voorde (10), who observed that whereas 10^3 cysts of *Naegleria* per cm^3 were sterilized by 2 mg of chlorine per liter in 30 min, 40 mg of chlorine per liter failed to sterilize acanthamoebic cysts.

It is realized that all experiments with chlorine discussed have been laboratory batch experiments, which differ considerably from the conditions found in thermal pools, lakes, rivers, and commercial pools. Continuous culture experiments that more closely represent such conditions are under way in our laboratory. Chlori-

TABLE 2. *Comparative amoebicidal capacities of chlorine dioxide, ozone, and deciquam 222*

Species (strain)	pH	Temp (°C)	Concn of amoebae × 10^4 per cm^3	ClO_2 concn (mg·liter^{-1})		No. of amoebae after 30 min	O_3 concn (mg·liter^{-1})		No. of amoebae after 30 min	Deciquam 222 concn (cm^{-3}·liter^{-1})	No. of amoebae after 30 min
				Initial	Final		Initial	Final		Initial	
N. gruberi (P1200f)	7.0	25	1.92	1.1	0.25	40	6.75	0.08	20	0.05	
				1.1	0.25	30				0.025	4
				1.0	0.25	40					
N. fowleri (MsT)	7.0	25	1.92	2.0	0.5	8	6.75	0.075	20	0.05	
				1.6	0.35	15				0.025	2
				1.3	0.25	20					
A. castellanii (1501)	7.0	25	1.92	3.4	0.75		6.75	0.078	20	0.05	
				2.9	0.65	1				0.025	6
				2.6	0.6	3					
A. culbertsoni (A-1)	7.0	25	1.92	3.4	0.75		6.75	0.08	0.05		
				2.5	0.6	1				0.025	2

TABLE 3. *Comparison of the properties of chlorine, chlorine dioxide, ozone, and deciquam 222*

Disinfectant	Disinfecting efficiency	Optimum pH	Optimum temp (°C)	Reaction with:					Toxicity	Solubility at 20 to 30°C	Reaction with UV[a]	Stability	Residual disinfectant	Cost
				Ca^{2+}, Mg^{2+}	NH_3	Organic N	Amino acids	Proteins						
Chlorine (Cl_2)	Good	2.0–7.0	22–25	–	+	+	+	+	High	Good	+	Low	Yes	Low
Chlorine dioxide (ClO_2)	Good (>Cl_2)	6.5–8.5+	30	–	–	+	–	+	Intermediate	Very good	+	High	Yes	High
Ozone (O_3)	Good (>Cl_2)	NK[b]	0–4	–	–	+	+	+	High	Very poor	–	Very low	No	Low
Quaternary ammonium compound (deciquam 222)	Very good (>Cl_2)	6.4–9.6	20–37	+	–	Very low	+	+	Low	Very good	–	High	Yes	High

[a] UV, Ultraviolet light.
[b] NK, Not known.

nation in practice involves breakpoint chlorination to establish and subsequently maintain an acceptable residual concentration (FAC) for disinfection. In New Zealand, this varies from 0.1 to 0.2 mg·liter^{-1} for potable water and from 0.5 to 0.8 mg·liter^{-1} for recreational waters. In a swimming pool situation, the more organic pollutants there are introduced by the bathers, the more FAC that will be needed for adequate disinfection. Thus, there is need for continual testing of the water to assess the fluctuating chlorine demand of the water. It is important to remember that chlorine, as well as the other disinfectants, will have an indirect effect on PFLA in potable waters by denying the amoebae their bacterial food source as most bacteria are destroyed at levels of 0.1 mg of chlorine per liter (24), thus starving the amoebae and forcing them to encyst. It has been shown that the cyst stage is noninfective (7).

Table 2 shows that chlorine dioxide is also an effective disinfectant against PFLA. Again, *Acanthamoeba* spp. were more resistant to the disinfectant than *Naegleria* spp. Because it forms a stable residual and is active at an alkaline pH, chlorine dioxide may be a valid alternative to chlorine, particularly in waters with a high ammonia content. Its main disadvantage is that it is easily expelled from solution and is highly explosive in its gaseous form (Table 3).

Because of the recent association of organohalides, formed in potable water by chlorination, with certain cancers, ozone is receiving more favorable attention. Its high germicidal properties and lack of toxic end products make it a viable alternative to chlorine (20). Under similar conditions, it is generally agreed that ozone is a more potent bactericide than chlorine, with concentrations as low as 0.04 mg of ozone per liter being effective against *Escherichia coli* (13).

Table 2 demonstrates that ozone is amoebicidal, but only at much higher concentrations than those of chlorine or deciquam 222. Table 2 also shows a high ozone demand, as reflected in the difference between the initial and final concentrations of ozone. No differences in sensitivity to ozone were detected between *Naegleria* spp. and *Acanthamoeba* spp.

The results in Table 2 show that deciquam 222 is a very potent amoebicide, being approximately 20 times more effective than chlorine. Its use has previously been reported by Das and Jadin (Int. Cong. Parasitol. 3rd, Munich, West Germany, ICP III, i:195–196, 1974) who obtained amoebicidal concentrations of 0.025 mg·liter^{-1}. This high amoebicidal activity is thought to be due to the extreme sensitivity of the amoebic plasma membrane to surface-active reagents

(3). Again, no differences in susceptibility between *Naegleria* spp. and *Acanthamoeba* spp. were observed. The relative bactericidal activity of quarternary ammonium compounds as compared to chlorine can be judged from the finding that 3.0 mg of cetyldimethylbenzylammonium chloride per liter was bactericidal for 2×10^6 coliforms within 15 min, whereas 8.0 mg of chlorine per liter failed to kill 9.8×10^5 coliforms in 30 min (23). The optimum pH range of quarternary ammonium compounds is usually between 6.4 and 9.6, and their biocidal activity is accelerated with increasing temperature (21). Quarternary ammonium compounds do not react with water, and therefore, there is no quarternary ammonium compound demand; because of this, possibilities for recycling exist (22). Quarternary ammonium compounds are, however, inactivated by hard waters.

Deciquam 222, chlorine, chlorine dioxide, and ozone have all been shown to possess disinfecting properties against PFLA, but concentrations higher than those used against bacteria are needed. Furthermore, the possession of resistant amoebic cysts complicates the disinfection process. Of the four disinfectants examined, deciquam 222 proved to be the most effective amoebicide, followed by chlorine, chlorine dioxide, and ozone. The final choice of a particular disinfectant, however, must remain tied to the physical and chemical parameters of the water to be disinfected. The necessity for an effective disinfectant can be judged by the increasing number of isolations of free-living amoebae from potable and recreational waters (2, 4, 5, 6, 14, 15). The majority of amoebae isolated after disinfection in this study belong to the genus *Acanthamoeba*, indicating the greater resistance of *Acanthamoeba* spp. to chlorine as compared with *Naegleria* spp. Striking an optimistic note, Lyons and Kapur (14) concluded, after a survey of 30 halogenated public swimming pools, that the low amoebic densities ($<1 \cdot liter^{-1}$) in the majority of pools illustrated that these organisms could be adequately controlled by proper pool maintenance.

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